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Reduction of Uranium(VI) Phosphate during Growth of the Thermophilic Bacterium *Thermoterrabacterium ferrireducens*

T. V. Khijniak,¹ A. I. Slobodkin,¹ V. Coker,² J. C. Renshaw,² F. R. Livens,²
E. A. Bonch-Osmolovskaya,¹ N.-K. Birkeland,³ N. N. Medvedeva-Lyalikova,¹
and J. R. Lloyd^{2*}

Winogradsky Institute of Microbiology, Russian Academy of Sciences, 7/2 Prospect 60-letiya Oktyabrya, Moscow 117312, Russia¹; The Williamson Research Centre for Molecular Environmental Science and The School of Earth, Atmospheric and Environmental Sciences, The University of Manchester, Manchester M13 9PL, United Kingdom²; and Department of Biology, University of Bergen, P.O. Box 7800, N-5020 Bergen, Norway³

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The thermophilic, gram-positive bacterium *Thermoterrabacterium ferrireducens* coupled organotrophic growth to the reduction of sparingly soluble U(VI) phosphate. X-ray powder diffraction and X-ray absorption spectroscopy analysis identified the electron acceptor in a defined medium as U(VI) phosphate [uramphite; $(\text{NH}_4)(\text{UO}_2)(\text{PO}_4) \cdot 3\text{H}_2\text{O}$], while the U(IV)-containing precipitate formed during bacterial growth was identified as ningyoite $[\text{CaU}(\text{PO}_4)_2 \cdot \text{H}_2\text{O}]$. This is the first report of microbial reduction of a largely insoluble U(VI) compound.

The capacity to enzymatically reduce U(VI) has been demonstrated for a range of Fe(III)-reducing and sulfate-reducing bacteria (8, 9). Mesophilic representatives of the genera *Geobacter*, *Shewanella*, and *Desulfotomaculum* are known to couple U(VI) reduction to growth, while *Desulfovibrio* species reduce U(VI) but do not obtain energy to support growth from this process (10, 14, 17, 18). Only a few thermophilic microorganisms have been shown to reduce U(VI) enzymatically, including *Thermus scotoductus*, *Pyrobaculum islandicum*, and *Thermoanaerobacter* sp. (5, 6, 15), but conservation of energy for growth during U(VI) reduction has not been demonstrated for any of these model organisms. In all of the studies mentioned above U(VI) was supplied in the form of soluble uranyl acetate, uranyl nitrate, or uranyl chloride, which form soluble U(VI)-carbonate complexes in bicarbonate-buffered medium.

Thermoterrabacterium ferrireducens is a thermophilic, gram-positive, anaerobic bacterium that is capable of organotrophic or lithoautotrophic growth with reduction of various electron acceptors, including Fe(III) (1, 2, 16). In order to determine the capacity of *T. ferrireducens* to reduce U(VI), fumarate-grown cultures (5%, vol/vol) were inoculated into anaerobic bicarbonate-buffered medium (pH 7.0) with glycerol (3 ml/liter) and yeast extract (0.2 g/liter; Sigma) as potential electron donors and uranyl acetate (2.5 mM; Aldrich-Sigma) as a potential electron acceptor. The medium composition and preparation protocols used were

those described previously (16), except that Fe(III) oxide was omitted. No reducing agent was added to the medium. Uranyl acetate was added by syringe to sterilized medium from a 50 mM stock solution. All experiments were performed in 27-ml Balch tubes with 10 ml of the medium incubated at 65°C in the dark without agitation. For uranium determination, 1-ml samples were withdrawn at regular intervals, and 0.5-ml aliquots were used for analysis. Cell pellets and precipitates were separated from supernatants by centrifugation ($10,000 \times g$, 5 min) and dissolved in 1 M HCl. U(VI) was quantified with 2-(5-bromo-2-pyridylazo)-5-diethylaminophenol at 578 nm (4). Samples were prepared in duplicate. Cells were counted by epifluorescence microscopy after acridine orange staining (3).

Immediately after injection of uranyl acetate into a test tube containing growth medium, a yellow precipitate formed. After 48 to 72 h of incubation, the color of the precipitates in the cultures of *T. ferrireducens* changed to gray, the U(VI) concentrations decreased, and significant bacterial growth was observed. The same processes repeatedly occurred after four subsequent 5% (vol/vol) transfers. No changes in precipitate appearance or U(VI) concentration were observed in abiotic control experiments (noninoculated medium). The yellow precipitate [analyzed immediately after addition of U(VI) or after 68 h of incubation in noninoculated medium] was identified by X-ray powder diffraction (XRD) analysis as uranium phosphate [uramphite; $(\text{NH}_4)(\text{UO}_2)(\text{PO}_4) \cdot 3\text{H}_2\text{O}$] (characteristic lines at 5.557, 3.799, 3.509, 3.279, 2.780, 2.179, 2.160, and 1.698 Å, with 40% or more intensity) (Fig. 1A), and the gray precipitate formed during bacterial growth on this solid-phase mineral was identified as ningyoite $[\text{CaU}(\text{PO}_4)_2 \cdot \text{H}_2\text{O}]$ (characteristic reflections corresponding to 4.333, 3.022, 2.809, 2.349,

* Corresponding author. Mailing address: The Williamson Research Centre for Molecular Environmental Science and The School of Earth, Atmospheric and Environmental Sciences, The University of Manchester, Manchester M13 9PL, United Kingdom. Phone: 44 161 275 7155. Fax: 44 161 275 3947. E-mail: jon.lloyd@manchester.ac.uk.

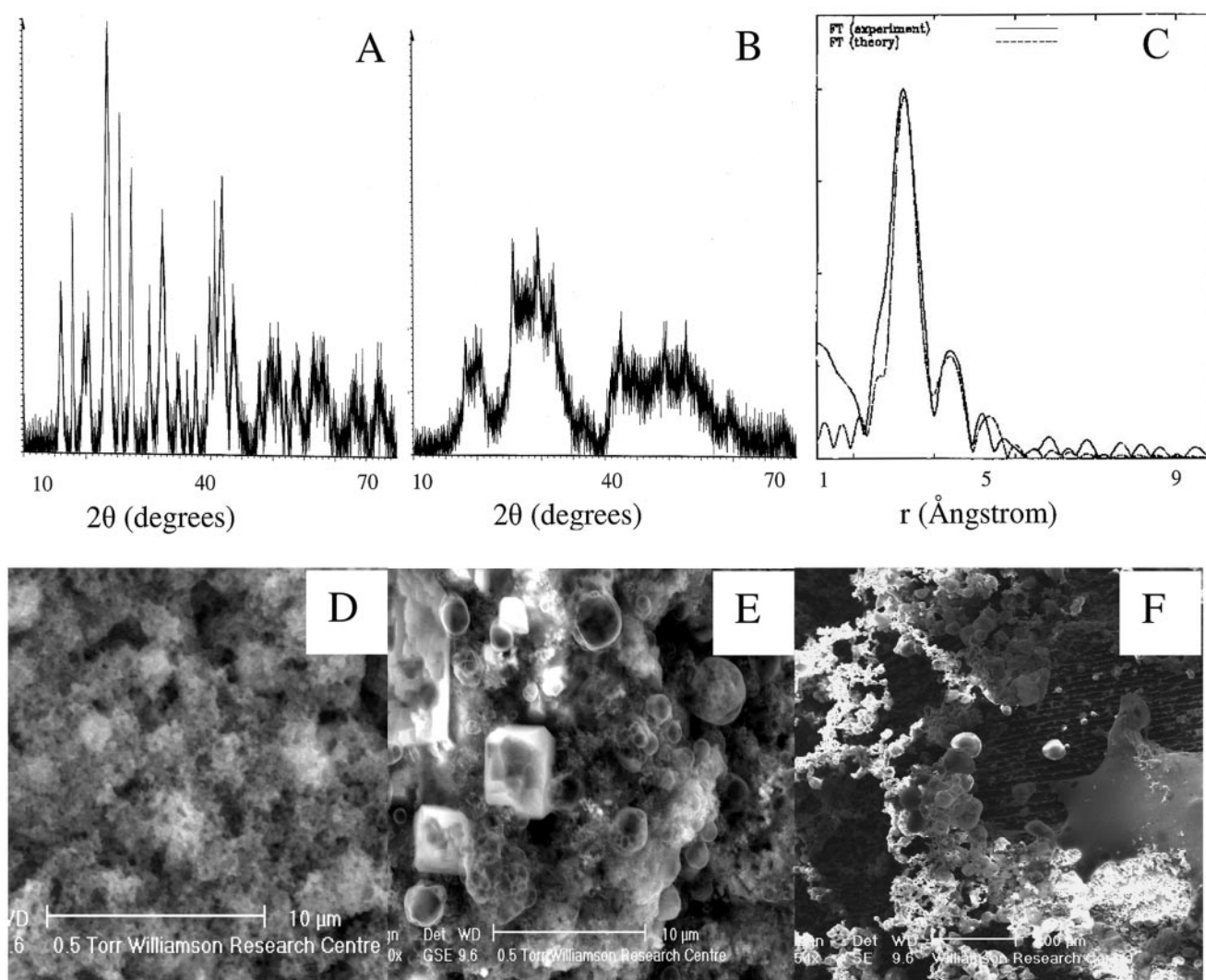


FIG. 1. XRD, EXAFS, and ESEM investigations of the initial yellow precipitate and the gray precipitate formed during growth of *T. ferrireducens*. (A and B) XRD analysis of yellow (A) and gray (B) precipitates. (C) EXAFS analysis of gray precipitates. (D and E) ESEM of air-dried yellow (D) and gray (E) precipitates. (F) ESEM cryostage analysis of gray precipitate.

2.128, 1.844, 1.736, and 1.691 Å (Fig. 1B). X-ray absorption near-edge spectroscopy carried out on station 16.5 at the Daresbury Synchrotron Radiation Source showed that the gray precipitate contained uranium in the tetravalent oxidation state, while extended X-ray absorption fine-structure spectroscopy (EXAFS) analysis confirmed the presence of coordinated phosphate (Fig. 1C). Environmental scanning electron microscopy (ESEM) of air-dried samples revealed that in noninoculated medium uranium phosphates and other elements formed a uniform mixture of crystals (Fig. 1D), while bacterial cultures contained round, uranium-containing particles (Fig. 1E). ESEM cryostage investigations performed to avoid possible artifacts during air drying confirmed the presence of round particles in the microbially formed precipitate (Fig. 1F).

Within 68 h, approximately 80 to 90% of the hexavalent uranium was reduced by growing cultures of *T. ferrireducens* when 1 or 2.5 mM U(VI) was added to the cultivation

medium (Fig. 2). No uranium reduction and no bacterial growth were detected with 5 or 10 mM U(VI), suggesting that there was toxicity at these higher concentrations. As evident from data for the U(VI) distribution between the liquid phase and precipitates, at a uranium concentration of 1 or 2.5 mM, almost all of the hexavalent uranium was precipitated by phosphate (2.4 mM PO_4^{3-} supplied in the medium) (Fig. 2). Thus, *T. ferrireducens* reduced U(VI) that was predominantly present as solid uramphite. Low concentrations of U(VI) present in the liquid were also reduced (Fig. 2).

T. ferrireducens used U(VI) as an electron acceptor during growth (Fig. 3). U(VI) reduction was coupled to an increase in the cell number, and the final cell yield was about 2.5-fold higher in the presence of U(VI) than it was in U(VI)-free controls. The degree of stimulation of growth by uranium was the same in basal medium and in medium prerduced with $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (final concentration, 0.5 g/liter; E_h ,

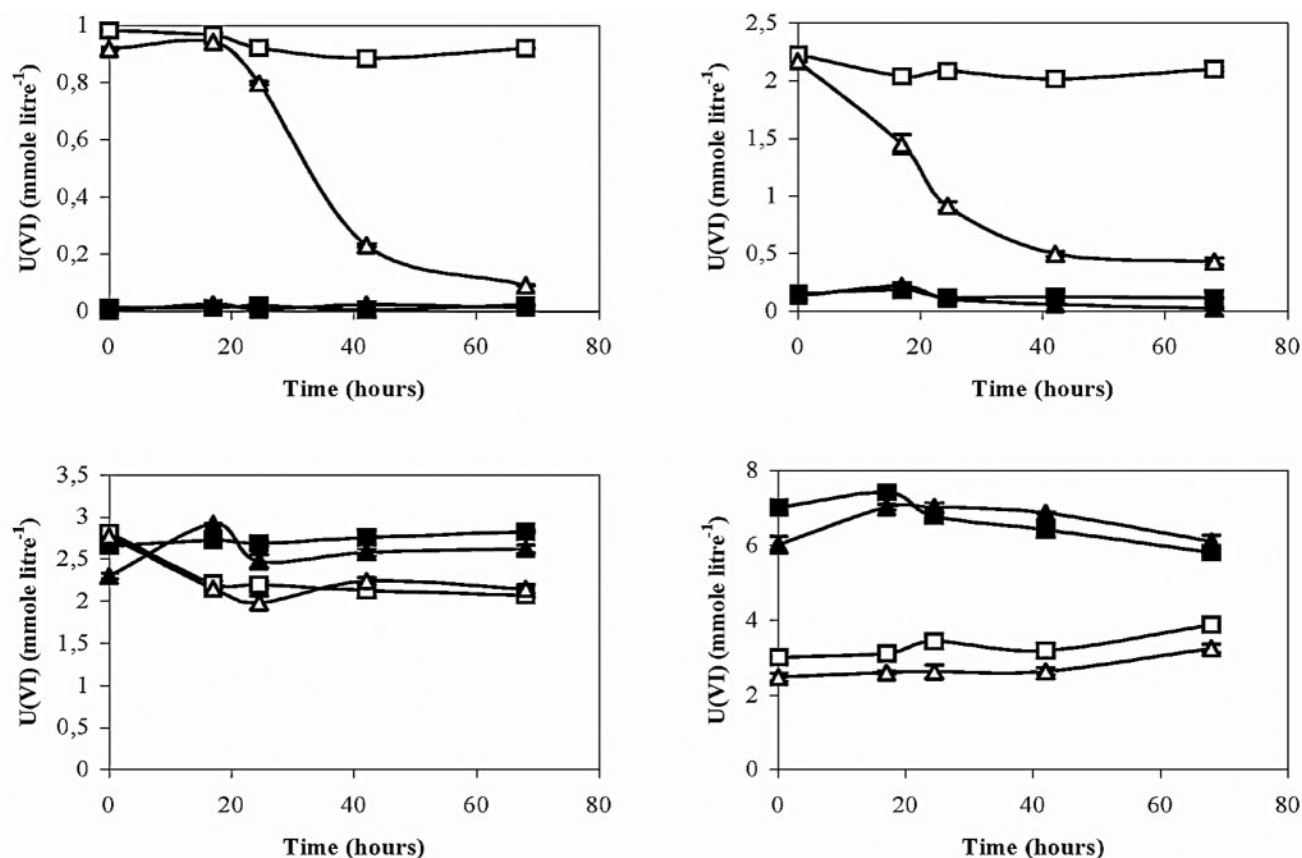


FIG. 2. Uranium reduction by *T. ferrireducens* with different initial U(VI) concentrations. Open symbols, U(VI) in precipitate; solid symbols, U(VI) in liquid; triangles, cultures of *T. ferrireducens*; squares, noninoculated medium.

< -110 mV), indicating that U(VI) reduction, and not the change in E_h due to tetravalent uranium formation, was responsible for the increased cell yields. Unlike the growth with other electron acceptors utilized by *T. ferrireducens*

[Fe(III) and fumarate], growth with U(VI) started after a lag phase of about 15 h.

In this work microbial reduction of a sparingly soluble uranium compound was demonstrated. Extracellular reduction

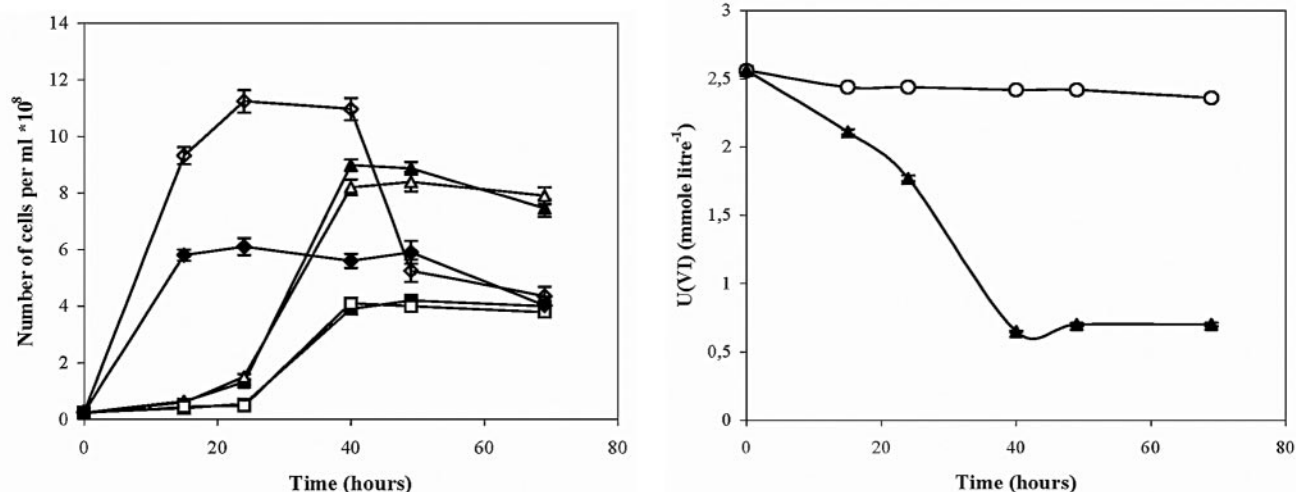


FIG. 3. Growth of and U(VI) reduction by *T. ferrireducens* under different cultivation conditions. Glycerol (30 mmol liter⁻¹) and yeast extract (0.2 g/l) were used as electron donors. Uramphite [2.5 mmol liter⁻¹ U(VI)], amorphous Fe(III) oxide (90 mmol liter⁻¹), and fumarate (20 mmol liter⁻¹) were used as electron acceptors. Solid triangles indicate cultures of *T. ferrireducens* incubated with U(VI) in basal medium. Symbols for left panel: \blacktriangle , U; \triangle , U + Na₂S; \blacksquare , no acceptor; \square , no acceptor + Na₂S; \diamond , Fe(III); \blacklozenge , fumarate. Symbols for right panel: \circ , control (no bacteria); \blacktriangle , *T. ferrireducens*.

has been established previously for transition metals [e.g., insoluble Fe(III) or Mn(IV) oxides] but not for actinide elements. Indeed, intensive studies of the mechanisms of electron transfer to Fe(III) or Mn(IV) (11, 12) have shown a range of mechanisms for reduction of these substrates, and some of these mechanisms may also be appropriate for U(VI) minerals. However, it has been reported that at uranium-contaminated sites, solid-phase U(VI) present in sediments is resistant to microbial reduction (13), although this form of U(VI) could be potentially bioavailable based on the results of our experiments. Furthermore, to date, the only product of microbial U(VI) reduction that has been reported is uraninite (UO₂) (7, 9), although our studies suggest that U(IV) calcium phosphate (ningyoite) can also be an end product of this form of microbial metabolism. In conclusion, this is the first report of a thermophilic microorganism (*T. ferrireducens*) conserving energy for growth via U(VI) reduction. Thus, the results of this study extend the limited number of known uranium-reducing microorganisms and demonstrate that there are biogenic transformations of U phosphate minerals that may take place in uranium ore deposits and uranium-contaminated environments.

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